

REMARKS

A Notice to Comply was issued Oct. 26, 2007, alleging that claims 23 and 24 do not read on the elected invention, and requiring that these claims be listed with the status identifier "Withdrawn." This change has been made, but the withdrawal of these claims is traversed.

The Notice indicated that Applicants' response of 28 August 2007 asserted that double stranded target polynucleotides (as found in claim 23) are encompassed by the claimed invention. This is incorrect. Claim 23 recites that the sample comprises double stranded target polynucleotide; this does not exclude the sample from also comprising single stranded target polynucleotide. RNA can form double stranded structures. Claim 23 thus reads on the elected invention. Applicants argued accordingly in their prior response.

Similarly, claim 24 recites that the target polynucleotide is produced by an amplification reaction. RNA can also be amplified. Exhibit A attached describes amplification of RNA. Claim 24 thus also reads on the elected invention.

Reconsideration is respectfully requested. The undersigned may be reached at the number below.

Respectfully submitted,

/David W. Maher/

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858-228-7829

**EXHIBIT A**

# Amplified RNA synthesized from limited quantities of heterogeneous cDNA

(cerebellum/guanine nucleotide-binding protein/T7 RNA polymerase/Purkinje cell)

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**ABSTRACT** The heterogeneity of neural gene expression and the spatially limited expression of many low-abundance messenger RNAs in the brain has made cloning and analysis of such messages difficult. To generate amounts of nucleic acids sufficient for use in standard cloning strategies, we have devised a method for producing amplified heterogeneous populations of RNA from limited quantities of cDNA. Whole cerebellar RNA was primed with a synthetic oligonucleotide containing the T7 RNA polymerase promoter sequence 5' to a polythymidylate region. After second-strand cDNA synthesis, T7 RNA polymerase was used to generate amplified antisense RNA (aRNA). Up to 80-fold molar amplification has been achieved from nanogram quantities of cDNA. The amplified material is similar in size distribution to the parent cDNA and shows sequence heterogeneity as assessed by Southern and Northern blot analysis. Specific messages for moderate-abundance mRNAs for actin and guanine nucleotide-binding protein (G-protein)  $\alpha$  subunits have been detected in the amplified material. By using *in situ* transcription to generate cDNA, sequences for cyclophilin have been detected in aRNA derived from single cerebellar tissue sections. cDNA derived from a single cerebellar Purkinje cell also has been amplified and yields material that hybridizes to cognate whole RNA and mRNA but not to *Escherichia coli* RNA.

The diversity of neuronal phenotypes presents a serious challenge to those studying gene regulation in the brain. While variation in levels of known mRNAs can be assessed by *in situ* hybridization (1) and *in situ* transcription (2) in selected brain regions, the identification and cloning of novel regulated messages from discrete neuronal populations has proved to be a formidable task. Obtaining sufficient mRNA for the isolation, cloning, and characterization of such regulated transcripts has been hindered by the high complexity of neural mRNA, the relatively low abundance of many important neuronally expressed messages, and the spatially limited expression of these messages.

The polymerase chain reaction (PCR) (3) is a powerful method for amplifying rare DNA species from tissue samples as limited as microdissected chromosomes (4). However, PCR requires both 5' and 3' sequence information for the synthesis of primers, thus precluding general amplification of all messages in a cell population. Modified PCR strategies, including homopolymeric tailing of the 3' terminus (5, 6) and synthesis of highly degenerate oligonucleotide primers (7), have been implemented recently to improve the range of cDNAs that can be cloned with PCR. Additional problems, however, curtail the usefulness of PCR for this task. Specifically, the widely used *Thermus aquaticus* (Taq) DNA poly-

merase has relatively low fidelity (8), and misincorporations are propagated through subsequent cycles of the amplification. The Taq polymerase also has difficulty transcribing sequences longer than 3 kilobases (kb) (J.H.E., unpublished observations), leading to a skewing of cDNA size towards smaller material with subsequent rounds of amplification.

We describe in this paper a technique for the amplification of broad classes of cDNAs. An RNA polymerase promoter is incorporated into each cDNA molecule by priming cDNA synthesis with a synthetic oligonucleotide containing the phage T7 RNA polymerase promoter. After synthesis of double-stranded cDNA, T7 RNA polymerase is added and antisense RNA is transcribed from the cDNA template. The processive synthesis of multiple RNA molecules from a single cDNA template results in amplified antisense RNA (aRNA), which may serve as starting material for cloning procedures using random primers. In this paper we describe the characteristics of aRNA generated from whole cerebellum, cerebellar tissue sections, and individual Purkinje cells and discuss some of the possible uses for this technique.

## MATERIALS AND METHODS

**Materials.** Avian myeloblastoma reverse transcriptase was obtained from Seikagaku Kogyo, Tokyo; RNase H, DNA polymerase I, T4 DNA polymerase, and all restriction enzymes were from Bethesda Research Laboratories; S1 nuclease was from Boehringer Mannheim; RNase block and T3 RNA polymerase were from Stratagene; T7 RNA polymerase (80 units/ $\mu$ l) was from Promega; and radioactive nucleotide triphosphates were from Amersham. Synthetic oligonucleotides were purified by acrylamide gel electrophoresis. The rat  $\beta$ -actin clone (9) was a gift of L. Kedes. pGEM-2 plasmids containing sequences encoding the  $\alpha$  subunit of stimulatory G<sub>s</sub>, regulatory G<sub>o</sub>, and inhibitory G<sub>i1</sub>, G<sub>i2</sub>, and G<sub>i3</sub> guanine nucleotide-binding proteins (G proteins) were gifts of R. Reed (10). IB15 cDNA (cyclophilin) was a gift of G. S. Sutcliffe (11).

**cDNA Synthesis.** Total RNA (40  $\mu$ g) isolated from rat cerebellum (12) was primed with 100 ng of primer (5'-AAA CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG CGC T<sub>15</sub>-3') as schematized in Fig. 1. The RNA/primer mix was subjected to three cycles of heat denaturation at 80°C alternating with incubation on ice for 3 min each. First-strand synthesis was performed with avian myeloblastoma virus reverse transcriptase, and second-strand cDNA was synthe-

Abbreviations: PCR, polymerase chain reaction; G protein, guanine nucleotide-binding protein; aRNA, amplified RNA; nt, nucleotide; IST, *in situ* transcription.

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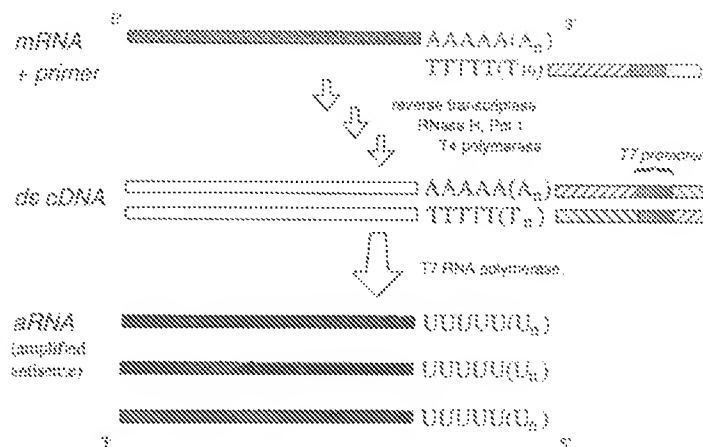


FIG. 1. Paradigm for production of aRNA. Whole RNA is reverse-transcribed by using a 57-nt synthetic primer containing the T7 RNA polymerase binding site (5'-AAA CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG GCG T<sub>15</sub>-3'). Second-strand cDNA synthesis [producing double-stranded (ds) cDNA] is performed with RNase H and *E. coli* DNA polymerase I (9). After cDNA is made blunt-ended with T4 DNA polymerase, the cDNA is purified and transcribed with T7 RNA polymerase, yielding antisense aRNA.

sized with RNase H and *Escherichia coli* DNA polymerase I (13). cDNA was made blunt-ended by treatment with 2 units of T4 DNA polymerase for 15 min at 37°C. Unincorporated triphosphates were removed by drop dialysis against double-distilled H<sub>2</sub>O for 2 hr with a 0.025-mm nitrocellulose filter (Millipore). Identical reactions were performed for each synthesis with and without incorporation of 30  $\mu$ Ci (1  $\mu$ Ci = 37 kBq) of [ $\alpha$ -<sup>32</sup>P]dCTP at 400 Ci/mmol.

**aRNA Synthesis.** For each reaction, 3 ng of cDNA was amplified in 40 mM Tris (pH 7.5) containing in a 20- $\mu$ l volume 6 mM MgCl<sub>2</sub>; 10 mM NaCl; 2 mM spermidine; 10 mM dithiothreitol; 500  $\mu$ M (each) ATP, GTP, and UTP; 12.5  $\mu$ M CTP; 30  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P]CTP (3000 Ci/mmol); 10 units of RNase block; and 80 units of T7 RNA polymerase. Reactions were carried out for 2 hr at 37°C. Incorporated radioactivity was determined by precipitation with CCl<sub>3</sub>COOH (14).

**In Situ Transcription (IST) and Amplifications.** Fresh frozen cerebellar tissue was cut by cryostat in a horizontal plane into 11- $\mu$ m-thick sections and processed for IST (2). The 57-nucleotide (nt) T7 RNA polymerase primer was hybridized for 12 hr and washed for 5 hr in 0.5 $\times$  SSC (1 $\times$  SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7) at room temperature, and IST was performed with 250  $\mu$ M of each dNTP (6). For localization, IST was performed on parallel sections with deoxycytidine 5'-[ $\alpha$ -(<sup>35</sup>S)]thiotriphosphate at 130 Ci/mmol. cDNA transcripts were isolated (2), second-strand cDNA was synthesized (14), and hairpin-loop structure was removed by S1 nuclease treatment. cDNA was made blunt-ended by using T4 DNA polymerase, was phenol/chloroform-extracted, was ethanol-precipitated with 5  $\mu$ g of carrier tRNA, and was drop-dialyzed against H<sub>2</sub>O for 4 hr in a 10- $\mu$ l volume. Two microliters was used for each aRNA amplification as described above, except that the concentration of nonlabeled CTP in the reaction was 1.25  $\mu$ M rather than 12.5  $\mu$ M.

**Single-Cell Injections and Amplifications.** Primary cultures of E20 rat embryo cerebellum (15, 16) consisting of Purkinje neurons, cerebellar interneurons, and glia were grown on polylysine in modified Eagle's medium with 10% added calf serum. At 21 days *in vitro*, Purkinje cells were impaled with polished, uncoated patch electrodes (3–5 Mohm) filled with 8–10  $\mu$ l of the reaction mixture consisting of 154 mM KCl, 6 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.1  $\mu$ M CaCl<sub>2</sub>, 10 mM Hepes (pH 7.3), reverse transcriptase at 2 units/ $\mu$ l, 57-nt T7 primer at 0.5 ng/ $\mu$ l, and 1 mM each of dATP, dCTP, dGTP and dTTP. Loading and incubation in the whole-cell recording configuration ranged from 5 to 20 min and allowed simultaneous monitoring of electrical properties of the cell.

Subsequently, cell soma were harvested with suction applied through the electrode holder after opening the electrode tip by gently touching it against the culture plate. Harvested soma were incubated individually in the electrodes for 1 hr at

37°C to facilitate first-strand synthesis, ejected from the electrodes into EDTA (10 mM) in two volumes of ethanol, and frozen on dry ice. tRNA (5  $\mu$ g) was added as carrier, and samples were phenol/chloroform-extracted and ethanol-precipitated. The nucleic acid was dissolved in 25  $\mu$ l of H<sub>2</sub>O and heated at 95°C for 3 min, followed by quick cooling on ice to separate the cDNA from RNA. To make second-strand cDNA, the volume was increased to 50  $\mu$ l with 2 $\times$  DNA polymerase buffer (14) and incubated with 10 units of DNA polymerase at 37°C for 60 min. This mixture was phenol/chloroform-extracted, followed by two precipitations with ethanol. The DNA was treated with 1 unit of S1 nuclease for 5 min (14), and the sample was phenol/chloroform-extracted and ethanol-precipitated. The cDNA was made blunt-ended by T4 DNA polymerase, which was followed by phenol/chloroform extraction, ether extraction, and drop dialysis against 50 ml of H<sub>2</sub>O for 4 hr. cDNA was concentrated to 10  $\mu$ l under vacuum; 3 to 5  $\mu$ l was used in each amplification reaction. aRNA amplification was done essentially as described above except that nonlabeled CTP was used at a concentration of 1.25  $\mu$ M and, when using 3  $\mu$ l of DNA template, <sup>32</sup>P-labeled CTP was increased to 50  $\mu$ Ci.

**Northern and Southern Analyses.** pCD vector encoding actin was digested with 10 units of *Pvu* II. Plasmids encoding G<sub>s</sub>, G<sub>o</sub>, G<sub>i1</sub>, G<sub>i2</sub>, and G<sub>i3</sub> proteins were digested respectively with *Eco*RI/*Bam*HI, *Eco*RI/*Eco*RV, *Eco*RI/*Xba*I, *Eco*RI/*Sau*96, and *Eco*RI/*Eco*RV, yielding DNA fragments that have been used as specific probes for individual G protein  $\alpha$  subunits (10). pCD-IB15 was digested with *Bam*HI (11). Ten micrograms each of the G protein- and actin-encoding plasmids and 5  $\mu$ g of pCD-IB15 were Southern-blotted as described (14). Gels (1.2% agarose/3% formaldehyde) of whole mouse brain and rat brain RNA (15  $\mu$ g) were transferred to nitrocellulose (14). Southern and Northern transfers were prehybridized overnight at 42°C in 50% formamide/6 $\times$  SSC containing 5 $\times$  Denhardt's solution (0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin) and 100  $\mu$ g of sheared, autoclaved salmon sperm DNA per ml. For hybridization, 2.5  $\times$  10<sup>6</sup> cpm of aRNA was added to the bag and incubated in the same buffer for 48 hr at 42°C. Blots were washed in 0.1 $\times$  SSC/0.2% sodium dodecyl sulfate (SDS) for 1 hr at 42°C and exposed to Kodak XAR film at -80°C with a Cronex intensifying screen (DuPont) for the times indicated in the figure legends.

## RESULTS

**General Characteristics of aRNA Amplification.** The general characteristics of the aRNA amplification were similar to those described by Melton *et al.* (17) for *in vitro* transcription from plasmid vectors with SP6 RNA polymerase. The degree of amplification obtained was strongly dependent on the

enzyme concentration and incubation time. Using the same amount of template (3 ng) and varying the amount of T7 RNA polymerase from 10 units to 80 units, we achieved a 4-fold improvement in yield of aRNA, from 30 ng to 120 ng. Maximum levels of amplification were obtained with incubation times between 2 and 4 hr; for incubations longer than 4 hr,  $\text{CCl}_3\text{COOH}$ -precipitable radioactivity began to decrease. In optimizing the reaction, we have found that increased UTP concentration (to 1 mM) increases aRNA synthesis, while inclusion of glycogen (used as a nonspecific carrier during cDNA precipitation) inhibits the aRNA reaction. Additionally, excess primer acts as an inhibitor of the reaction, while microgram amounts of tRNA used as carrier can produce nonspecific RNA synthesis with high levels ( $>100$  units) of T7 RNA polymerase.

To assess the role of additional sequence located 5' to the T7 promoter site in aRNA production (potentially necessary to stabilize the enzyme-DNA interaction), we performed amplifications with synthetic primer lengths of 38, 57, and 80 nt, identical except for additional 5' sequence derived from pBluescript plasmid. Although cDNA generated from all three primers amplified equally well, the 57-nt primer gave the best yield of cDNA and was used for all subsequent amplification reactions. Additionally, we have amplified cDNA primed with a T3 RNA polymerase promoter site [55 nt long containing a 3' poly(dT) tract of 15 bases] with T3 RNA polymerase. This promoter-polymerase combination generated a similar size distribution of amplified material but yielded  $\approx 75\%$  of  $\text{CCl}_3\text{COOH}$ -precipitable radioactivity as compared with equal units of T7 RNA polymerase (data not shown). Using cDNA synthesized from total cerebellar RNA with the 57-nt T7 primer, we achieved 80-fold molar amplification from nanogram quantities of cDNA as measured by  $\text{CCl}_3\text{COOH}$ -precipitable radioactivity.

**Amplification of a Broad Range of mRNA Sequences from Total Cerebellar RNA.** Because the 5' promoter sequence is specific for T7 RNA polymerase (18, 19) and the RNA polymerase is capable of producing transcripts of 7 kb or larger (17), we expected the aRNA produced to represent accurately the size and complexity of the synthesized cDNA. To test this, we synthesized cDNA from total cerebellar RNA using the 57-nt T7 primer. A nonlabeled portion of this cDNA was then amplified with T7 RNA polymerase in the presence of  $[\alpha\text{-}^{32}\text{P}]\text{CTP}$ . The size distribution of cDNA and that of aRNA were very similar (Fig. 2 *Left*).

To further characterize the aRNA, we used aRNA to probe a Northern blot of whole-rat-cerebellar RNA and whole-mouse-brain RNA (Fig. 2 *Right*). The aRNA bound to a wide range of sequences, indicating sequence heterogeneity in the amplified material; the aRNA also showed a higher affinity for the cognate-species RNA. Approximately 30% of the total grain density of the bound material was found in the 18S and 28S ribosomal bands. This is consistent with the yield of cDNA corresponding to these RNA species when cDNA is made from total RNA, suggesting that aRNA abundance is representative of the parent cDNA. In similar experiments, aRNA showed broad complexity and marked species specificity when used to probe Southern blots of mouse and rat genomic DNA (data not shown). Attempts to assay complexity by RNA-RNA  $R_{\text{CT}}$  analysis were unsuccessful because of high levels of RNase A-resistant material in the single-stranded aRNA, suggesting significant secondary and tertiary structure under the hybridization conditions used (20).

**Detection of Specific Sequences in aRNA Synthesized from Nanogram Quantities of cDNA.** To detect and characterize regulated transcripts, an amplification method must be able to amplify moderate and low-abundance transcripts. To detect such transcripts, we generated aRNA at a specific activity of  $5 \times 10^8$  cpm/ $\mu\text{g}$  from 3 ng of cerebellar cDNA primed with the 57-nt T7 primer;  $2.5 \times 10^6$  cpm was used to

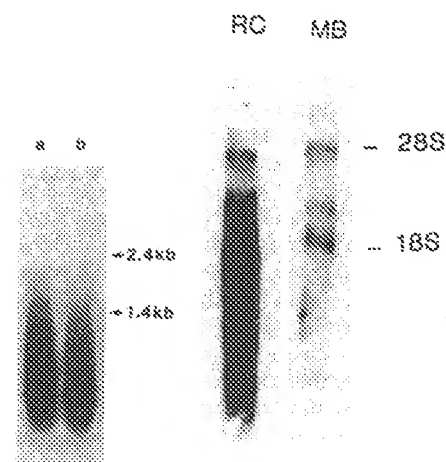


FIG. 2. (*Left*) Size distribution of aRNA compared with parent cDNA. Equal cpm of  $[\text{P}^{32}]\text{cDNA}$  (lane a) and  $[\text{P}^{32}]\text{aRNA}$  (lane b) were electrophoresed on a 1% agarose/3% formaldehyde gel. Size standards were provided by an RNA ladder (BRL). (*Right*) Complexity and sequence specificity of aRNA. Northern blot of 1% agarose/3% formaldehyde electrophoresis gel of 10  $\mu\text{g}$  of rat cerebellum RNA (lane RC) and 10  $\mu\text{g}$  of mouse brain RNA (lane MB), probed with  $2.5 \times 10^6$  cpm of  $[\text{P}^{32}]\text{CTP}$ -labeled aRNA derived from rat cerebellar cDNA and washed at high stringency. Size standards were provided by ribosomal RNA.

probe cDNAs encoding rat actin and G-protein  $\alpha$  subunits, with vector sequences in each lane serving as an internal nonspecific hybridization control. After filters were washed in  $0.1 \times \text{SSC}/0.1\%$  SDS at  $42^\circ\text{C}$ , strong signals were visible in the actin and  $G_s$  lanes (Fig. 3). In this figure other G-protein mRNAs are not visible with this exposure time; however, since the  $G_i$  and  $G_o$  protein  $\alpha$  subunits share considerable

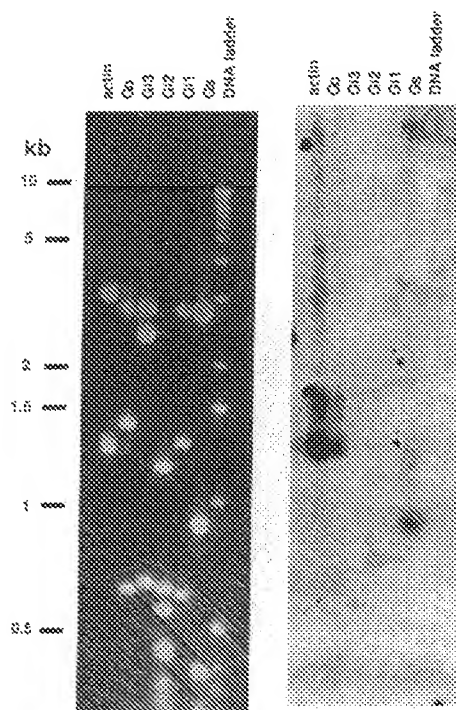


FIG. 3. Detection of specific sequences in aRNA from whole cerebellum. (*Left*) Ethidium bromide-stained 1% agarose gel of restriction enzyme digests of the plasmids encoding actin (*Pvu* II),  $G_o$  (*Eco*RI/*Eco*RV),  $G_{i3}$  (*Eco*RI/*Eco*RV),  $G_{i2}$  (*Eco*RI/*Sau*96),  $G_{i1}$  (*Eco*RI/*Xba* I),  $G_s$  (*Eco*RI/*Bam*HI), and the DNA 1-kb ladder. (*Right*) Southern blot of the same gel probed with  $2.5 \times 10^6$  cpm of  $[\text{P}^{32}]\text{CTP}$ -labeled aRNA derived from whole-rat-cerebellum cDNA.

homology, this may be due to dilution of the aRNA for these G-protein  $\alpha$  subunits among the multiple DNA restriction fragments encoding these sequences. To test this possibility, we also assayed for the presence of particular messages in the aRNA by probing slot-blots of nanogram amounts of aRNA and parent cDNA with random hexamer-primed probes for actin and the G proteins. Qualitatively identical results were found for both cDNA and aRNA: a strong signal was discerned for the actin probe in both aRNA and cDNA, a weaker signal was observed for G<sub>s</sub>, and weak signals were discerned for G<sub>o</sub>, G<sub>i1</sub>, and G<sub>i3</sub> (data not shown).

**Detection of Specific aRNA Transcript from Cerebellar Tissue Sections.** To assess the usefulness of aRNA amplification for producing increased amounts of nucleic acids from cDNAs transcribed directly from tissue sections, we performed IST (2) on cerebellar sections using the 57-nt T7 primer and then amplified these transcripts to aRNA. The IST autoradiograph signal (Fig. 4A) suggests that cDNA synthesis occurred in many cell types, with high signal density in the granule cell layer of the cerebellum. This result reflects the expected increase in the amount of mRNA in regions of high cellular density. The aRNA produced from IST-generated cDNA contains sequences for IB15 (band at  $\approx 680$  nucleotides) (Fig. 4B). Background is minimal as shown by the lack of hybridization of aRNA to the pCD vector in the IB15 sample and the lack of binding to the DNA ladder.

**Characterization of Amplification Products from a Single Cell.** A schematic of the microinjection technique used to introduce the 57-nt T7 primer into individual Purkinje cells is presented in Fig. 5A. The amount of radiolabel incorporated into aRNA obtained from single-cell amplifications from individual Purkinje cells ranged from 30,000 to 300,000 cpm. The reasons for this variability are unclear but may result from different amounts of first-strand cDNA synthesis, variable success in regeneration of functional T7 RNA polymerase promoter site during the preparation of the cDNA template, or partial degradation of the 57-nt T7 primer (21). This low number of radioactive cpm precluded the use of single-cell aRNA as a probe to screen for specific low-abundance mRNAs yet was sufficient to serve as a probe for cerebellar RNA to gauge the complexity of the amplified material. The aRNA probe is shown in Fig. 5C to hybridize to poly(A)<sup>+</sup> (lane 3) and total (lane 4) RNA isolated from the rat cerebellum. The specificity of this RNA signal is evident in the

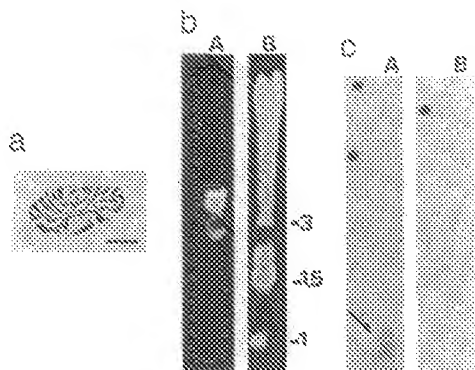


FIG. 4. Detection of specific sequences in aRNA derived from a single tissue section by *in situ* transcription. (a) Autoradiogram generated from a rat cerebellum tissue section when using the 57-nt T7 primer and incorporating deoxycytidine 5'-[ $\alpha$ -(<sup>35</sup>S)]triphosphate into the *in situ* transcription reaction. (Bar = 0.25 cm.) (b) Ethidium bromide-stained 1% agarose gel of 5  $\mu$ g of BamHI-digested pCD containing IB15 (lane A), and DNA ladder (lane B). (c) Southern blot of this gel, probed with  $1 \times 10^6$  cpm of [<sup>32</sup>P]CTP-labeled aRNA derived from an unlabeled *in situ* transcription of a rat cerebellum tissue section.

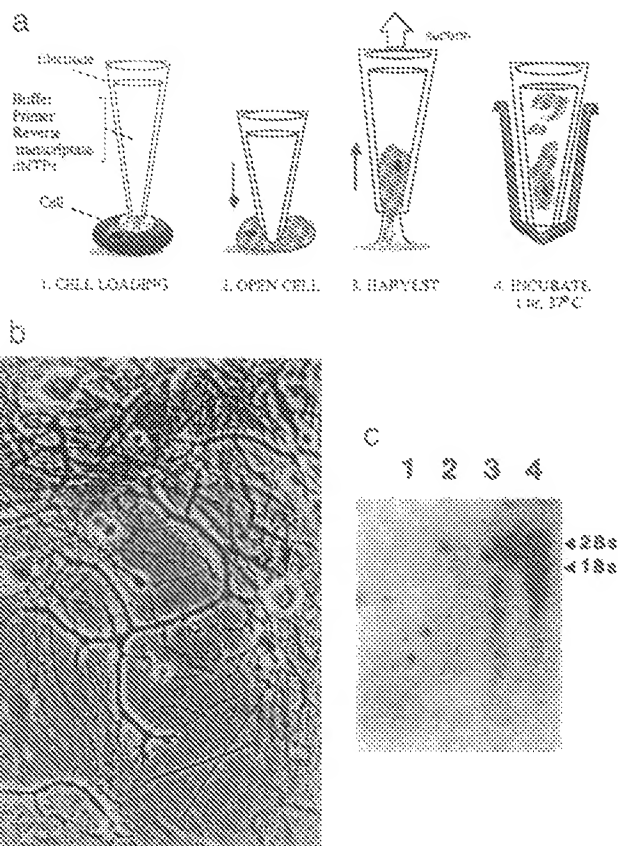


FIG. 5. (a) Diagram of cell-loading technique. The components for first-strand cDNA synthesis were loaded intracellularly by passive perfusion of the reaction mix with recording saline from the patch electrode; whole-cell recording configuration of the patch-clamp technique was used. (b) Phase-contrast micrograph of a mature Purkinje cell (arrow at soma) at 21 days in culture, identified by the characteristic dendritic arborization and relatively large size. The identification of Purkinje neurons was corroborated by characteristic functional properties, such as sustained spontaneous firing at  $>10$  Hz. (Bar = 10  $\mu$ m.) (c) Northern blot of 5  $\mu$ g of *E. coli* RNA (lane 1), 1  $\mu$ g of pBluescript (lane 2), 1  $\mu$ g of cerebellar poly(A)<sup>+</sup> RNA (lane 3), and 5  $\mu$ g of cerebellar total RNA (lane 4). This Northern blot was probed with  $3 \times 10^5$  cpm of aRNA derived from amplification of single-cell cDNA. After the blot was washed, it was exposed to x-ray film for 1 week with Cronex intensifying screen at  $-80^\circ\text{C}$  prior to development.

lack of hybridization of aRNA to *E. coli* total RNA (lane 1) and pBluescript DNA (lane 2).

## DISCUSSION

The high degree of specificity shown by T7 RNA polymerase for its promoter site (19) has made this enzyme a useful reagent in a variety of techniques, including *in vitro* RNA synthesis from plasmids containing the promoter site for use as probes (17), for *in vitro* translation studies (22), and for use in producing synthetic oligoribonucleotides (23). Sarkar and Sinner (24) recently utilized RNA amplification in conjunction with PCR to detect extremely low-abundance messages in heterologous cell types: once PCR had been carried out for a number of rounds, the phage RNA polymerase was used to generate single-stranded material for sequencing. The technique described in this paper differs from that of Sarkar and Sinner in that we have used T7 RNA polymerase to amplify a broad spectrum of messages without knowledge of sequence.

aRNA utilizes the specificity of the T7 RNA polymerase promoter site to allow *in vitro* amplification of a heteroge-



neous, complex population of cDNA. This methodology is capable of replicating a broad range of cDNAs without prior cloning into vectors. Although the optimization of all parameters for this method is not yet complete, our current degree of amplification is equivalent to that obtained with linearized plasmid containing the T7 promoter site. The degree of amplification may possibly be improved by using a higher concentration of RNA polymerase; in pilot experiments using 1000 units of enzyme per reaction, a 3- to 5-fold increase in CCl<sub>3</sub>COOH-precipitable aRNA was achieved compared to our established protocol using 80 units of enzyme.

Our results indicate that the spectrum of aRNA produced during amplification qualitatively reflects the population of cDNA from which it is produced. Since relative amounts of individual sequences present in cDNA approximate their relative abundances in the transcribed RNA population, the amount of specific RNA in an aRNA population should reflect its abundance in the original RNA population. Given this, our results from both aRNA-probed Southern blots and slot-blots of aRNA and cDNA probed with actin and G-protein cDNAs suggest that actin mRNA is considerably more abundant in rat cerebellum than any of the G-protein  $\alpha$ -subunit mRNAs tested. Of greater interest is the finding that G<sub>s</sub>  $\alpha$  subunit produced a substantially higher hybridization signal than did signals from the other G  $\alpha$ -subunit sequences tested. This is surprising because G<sub>s</sub>  $\alpha$ -subunit levels are lower than those of other G  $\alpha$  subunits in a variety of tissues (25), including brain (26). Such high relative abundance of G<sub>s</sub>  $\alpha$ -subunit mRNA has been detected, however, in other brain regions by *in situ* hybridization (27).

A number of additional applications are possible for this technology. First, aRNA may be a useful intermediate for construction of cDNA libraries from extremely limited amounts of tissue, such as individual brain nuclei, tissue sections, and potentially single cells. Second, with appropriate amplification primers, aRNA synthesis can be used for the production of specific ribonucleotide probes without prior cDNA cloning into vector or for the introduction of directionally cloned material with RNA polymerase promoters into vectors that lack such sequences. Third, aRNA may provide a source of large amounts of single-stranded, antisense material for use as the driver in subtractive hybridization (28). The specificity of bacteriophage RNA polymerase promoters also suggests a novel subtractive hybridization paradigm. aRNA produced from RNA population no. 1 (e.g., from 57-nt T7-generated cDNA) could be hybridized with RNA population no. 2. Unhybridized poly(A)<sup>+</sup> RNA could then be reverse-transcribed into cDNA by using a different RNA polymerase promoter-primer (i.e., T3 or SP6). After second-strand synthesis, aRNA could be synthesized by adding the appropriate RNA polymerase, thus yielding a high-specific-activity single-stranded probe for use in screening libraries.

Although the aRNA paradigm should provide a useful adjunct to PCR in a variety of studies, we believe it will especially facilitate studies of neural gene expression. Identification of mRNAs that vary as a function of arousal state, behavior, drug treatment, and development, for example, has been hindered by both the difficulty in construction of cDNA libraries from small brain nuclei and in the relative spatial insensitivity of subtractive hybridization techniques. Use of the aRNA amplification method in construction of cDNA libraries from individual brain nuclei potentially will provide a greater representation of low-abundance mRNAs from these tissues compared with their representation in whole-brain cDNA libraries and may facilitate cloning of important low-abundance messages. With further optimizations of aRNA synthesis and refinement of the whole-cell patch-clamp technique, it may be possible to create cDNA libraries

from single cells by using an aRNA intermediate, permitting cloning and analysis of important developmentally or functionally regulated transcripts from identified cell types within heterogeneous, complex cell populations.

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